CHAPTER 20

The Use of *Xenopus* Egg Extracts to Study Mitotic Spindle Assembly and Function *in Vitro*

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I. Introduction
II. Preparation of CSF Extracts for Spindle Assembly
    A. Requirements for Extract Preparation
    B. Protocol for Extract Preparation
    C. General Considerations for Preparing Spindle Assembly Extracts
    D. Procedure for Extract Preparation
    E. Preparation and Use of Sperm Nuclei and Fluorescent Tubulin
III. Spindle Assembly Reactions
    A. General Considerations for Spindle Assembly Reactions
    B. Reagents Required for Spindle Assembly
    C. CSF Spindle Assembly
    D. Cycled Spindle Assembly
IV. Monitoring Spindle Assembly Reactions
    A. Methods for Pelleting Spindles onto Coverslips
    B. Time-Course Experiments
V. Manipulation of Extracts
    A. Immunodepletion of Extracts
    B. Reagent Addition to Extracts
VI. Data Analysis and Interpretation
VII. Anaphase *in Vitro*
    A. Preparation of Anaphase-Competent Extracts
    B. Setting Up and Monitoring Anaphase Reactions
    C. Real-Time Analysis of Anaphase *in Vitro*
    D. Manipulation of Anaphase *in Vitro*

METHODS IN CELL BIOLOGY, VOL. 61

0091-679X/99 $30.00

VIII. Conclusions
References

I. Introduction

Since Flemming first described mitosis more than a century ago, understanding the mechanisms underlying cell division has been a major focus in cell biology (Flemming, 1965). Over the years, the study of cell division has evolved from a detailed description of mitosis by the early cytologists to a modern molecular investigation. Essential for this evolution has been the development of experimental systems in which molecular and observational studies can be linked. Extracts from unfertilized *Xenopus* eggs provide one such system. These extracts are capable of maintaining specific cell cycle states and carrying out many of the events associated with cell division *in vitro* (Lohka and Masui, 1983; Blow and Laskey, 1986; Hutchison *et al.*, 1988; Murray and Kirschner, 1989). In addition to providing fundamental insights into the nature of the cell cycle, these extracts have been very useful for dissecting downstream mitotic processes, such as regulation of microtubule dynamics, chromosome condensation, mitotic spindle assembly, sister chromatid separation, anaphase chromosome movement, and kinetochore assembly (Lohka and Maller, 1985; Belmont *et al.*, 1990; Verde *et al.*, 1990; Hirano and Mitchison, 1991; Sawin and Mitchison, 1991; Shamu and Murray, 1992; Murray *et al.*, 1996; Desai *et al.*, 1997, 1998). A hallmark of studies in *Xenopus* extracts has been the combination of detailed structural and functional analysis of complex macromolecular assemblies such as the mitotic spindle with the manipulation of selected components. Similar feats have been difficult to accomplish in genetic systems such as budding yeast because the cytology is limiting and the biochemistry is difficult. Furthermore, *Xenopus* extracts also provide insight into the mechanisms of vertebrate mitosis, the details of which may not be revealed from studies in lower eukaryotes.

The two most useful attributes of *Xenopus* extracts are their ability to maintain specific cell cycle states and to recapitulate many of the detailed morphological changes associated with mitosis. Both of these features depend on the natural cell cycle arrest of the frog egg and on the ability to make concentrated extracts that retain many of the properties of intact cytoplasm. Mature *Xenopus* eggs are arrested in metaphase of meiosis II by an activity termed cytostatic factor (CSF), which is thought to be the product of the c-*mos* protooncogene (Sagata *et al.*, 1989). Sperm entry triggers a calcium spike that initiates a series of events leading to the destruction of CSF and exit from the meiosis II metaphase arrest. This calcium sensitivity of the CSF arrest is exploited in the preparation of extracts by use of the calcium chelator EGTA. The presence of EGTA in buffers results in extracts that maintain the CSF arrest (referred to as CSF extracts) but can be induced to exit the CSF arrest by addition of calcium (Lohka and Maller,

1985). This convenient control of cell cycle state allows one to easily obtain *in vitro* spindles with replicated chromosomes as described later in detail. It should be noted that CSF extracts are meiotic (meiosis II) and not mitotic cytoplasm, a distinction that has not generally been made in the literature. However, given the mechanistic similarity of meiosis II and mitosis, studies using CSF extracts should be generally relevant to the study of mitosis, and several phenotypes of depletion of spindle assembly components in CSF extracts have also been observed in antibody-injection experiments in somatic cells (Sawin *et al.*, 1992; Blangy *et al.*, 1995; Gaglio *et al.*, 1995, 1996, 1997; Merdes *et al.*, 1996; Heald *et al.*, 1997) as well as with genetic analysis of mitotic mutants (Enos and Morris, 1990; Hagan and Yanagida, 1990; Hoyt *et al.*, 1992; Roof *et al.*, 1992).

In this chapter, we will present detailed methods for the preparation of CSF extracts and for performing spindle assembly reactions. We will also describe methods for depleting specific components from extracts, an approach that has been used successfully to determine the contributions of both motor and nonmotor components to spindle assembly (Sawin *et al.*, 1992; Merdes *et al.*, 1996; Walczak *et al.*, 1996, 1997). Finally, we will describe methods for analyzing anaphase *in vitro* (Murray *et al.*, 1996). We recommend that anyone interested in using *Xenopus* egg extracts as an experimental system should first consult Murray (1991) who provides an introduction to the early stages of the *Xenopus* life cycle, documents the history of cell cycle extracts, provides detailed technical descriptions on the preparation of different types of extracts, and gives advice on troubleshooting problems with extracts; Murray's article serves as the basis for several of the procedures described here.

II. Preparation of CSF Extracts for Spindle Assembly

A. Requirements for Extract Preparation

1. Buffer and Reagent Stocks

10× MMR
50 m*M* Na–Hepes, pH to 7.8 with NaOH
1 m*M* EDTA
1 *M* NaCl
20 m*M* KCl
10 m*M* $MgCl_2$
20 m*M* $CaCl_2$
Autoclave and store at room temperature (RT); if desired, MMR can also be prepared as a 25× stock

20X XB salts
2 *M* KCl
20 m*M* $MgCl_2$

2 m*M* $CaCl_2$
Sterile filter and store at 4°C

2 M Sucrose
Sterile filter or autoclave and store at 4°C or in aliquots at −20°C

1 M K-Hepes
pH to 7.7 with KOH, sterile filter and store at 4°C or in aliquots at −20°C

0.5 M K-EGTA
pH to 7.7 with KOH, sterile filter and store at RT

1 M $MgCl_2$
Sterile filter and store at RT

20X energy mix
150 m*M* creatine phosphate
20 m*M* ATP
20 m*M* $MgCl_2$
Store in 100-μl aliquots at −20°C

Protease inhibitors (LPC)
10 mg/ml each of leupeptin, pepstatin A, and chymostatin dissolved in DMSO; store in 100-μl aliquots at −20°C

Cytochalasin B or D
10 mg/ml in DMSO; store in 10- or 50-μl aliquots at −20°C
(Note: Both protease inhibitors and cytochalasin stocks can be frozen and thawed multiple times without detriment)

5% Gelatin (w/v)
Dissolved in water; autoclave and store in aliquots at −20°C

Cysteine, free base
Sigma No. C-7755

Versilube F-50
ANDPAK-EMA

Hormone stocks for priming frogs and inducing ovulation
Pregnant mare serum gonadotropin (PMSG): 100 U/ml (Calbiochem No. 367222) made up in water and stored at −20°C
Human chorionic gonadotropin (hCG): 1000 U/ml (Sigma No. CG-10) made up in water and stored at 4°C
(Hormones are injected into the dorsal lymph sac using a 27-gauge needle)

2. Equipment

Glass petri dishes
60-, 100-, 150-mm-diameter regular and 150-mm-diameter high-sided, i.e., 150-mm diameter and 75-mm height

13 X 51-mm Ultraclear tubes (Beckman No. 344057)

Ultracentrifuge and SW50.1/SW55.1 rotor
Clinical centrifuge

3. Primed Frogs

Frogs for extract preparation are primed using progesterone (present in PMSG) which induces maturation of oocytes. Priming is performed in two steps by injection with 50 U of PMSG (0.5 ml of 100 U/ml stock) on Day 1 and 25 U of PMSG (0.25 ml) on Day 3. Primed frogs are stored in dechlorinated water containing 2 g/liter rock salt in a cool room (ambient temperature of 16–20°C) and can be induced to lay eggs for up to 2 weeks after the second priming.

B. Protocol for Extract Preparation

1. Day before Extract Preparation

Sixteen to 18 h before extract preparation, four to six primed frogs are induced to ovulate by injection with 500 U (0.5 ml) of hCG. After injection, frogs are rinsed well in distilled water, put individually into plastic buckets (Fisher No. 03-484-21) containing two liters MMR, and stored overnight in a 16°C incubator.

2. Setup for Extract Preparation

For a four to six frog prep, you will need the following prior to beginning the prep:

a. Buffers

Prepare just before use unless indicated otherwise; all graduated cylinders and glassware should be rinsed well with double-distilled water (dd H_2O) prior to use.

Two or three liters 1X MMR (the MMR can be made the night before and stored in a plastic carboy in the 16°C incubator)

200 ml dejellying solution: 2% (w/v) cysteine in 1X XB salts; pH to 7.8 by adding 0.9 ml of 10 *N* NaOH

750 ml XB: 10 m*M* K-Hepes (pH 7.7), 100 m*M* KCl, 1 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, and 50 m*M* sucrose; prepare using 20X XB salts, 2 *M* sucrose, and 1 *M* K-Hepes (pH 7.7) stocks; to maintain a pH of 7.7 at 10 m*M* Hepes, add 11 μl 10 *N* KOH per 100 ml XB

250 ml CSF-XB: XB + 1 m*M* $MgCl_2$ + 5 m*M* EGTA; to prepare, transfer 250 ml of XB (from the 750 ml prepared previously) to a separate graduated cylinder and add EGTA to 5 m*M* and $MgCl_2$ to 1 m*M* final

100 ml CSF-XB + PIs: CSF-XB + 10 μg/ml LPC; to prepare, transfer 100 ml of CSF-XB (from the 250 ml prepared previously) to a separate graduated cylinder and add LPC to 10 μg/ml (mix immediately after adding LPC)

b. Equipment, etc.

SW55.1 or SW50.1 rotor at 16°C in ultracentrifuge

Two Pasteur pipettes with tips broken and fire polished to ~3 or 4-mm diameter

Four to six 13 × 51-mm Ultraclear tubes

Two 150-mm regular petri dishes, one high-sided 150-mm petri dish; rinse well with dd H_2O and coat with 100 μg/ml gelatin (thaw gelatin at 37°C, pour ~30 ml MMR into petri dish, pipet in 60 μl of gelatin and swirl well for ~30 sec, and pour out MMR and replace with XB); if making a squeezed egg extract in parallel (see Section VII,A) gelatin coat four 60-mm petri dishes (15 ml MMR and 30 μl gelatin) and fill with MMR for squeezing frogs into dishes, and coat one 100-mm petri dish for processing the squeezed eggs after dejellying

600-ml glass beaker (rinsed well with dd H_2O)

C. General Considerations for Preparing Spindle Assembly Extracts

Spindle assembly is best performed using freshly prepared extracts because freezing extracts considerably decrease their ability to form bipolar spindles. For spindle assembly extracts, egg quality must take precedence over egg quantity. Because 1 or 2 ml of extract is more than sufficient for most experiments, it is best not to use any batches of eggs containing significant numbers (>10%) of lysed eggs, activated eggs (detected by contraction of dark pigment at the animal pole), or "puffballs" (eggs which are swollen and often white and puffy). Strings of eggs which do not have fully separated jelly coats can be used as a last resort unless they contain a high percentage of deformed or activated eggs. Any batches of eggs which are distinguishable in quality should be processed separately. Although the best spindle assembly extracts are often made from freshly squeezed eggs, we have had good success with laid eggs and will focus here on their use. Further discussion of squeezed egg extracts is presented in Section VII,A.

For laid eggs, extract preparation should begin 16–18 hr after hCG injection. We recommend that the temperature of the buffers and the room in which eggs will be manipulated not exceed 23°C (ideally 18–20°C). Warmer temperatures will almost certainly result in lower quality extracts. Rinsing of eggs after dejellying is best performed in gelatin-coated petri dishes by swirling the dishes a couple of times and pouring out the buffer. Never pour solutions directly onto the dejellied eggs because they are quite fragile. Pour the solutions down the side of the wash vessel, and then gently swirl the eggs. Maximal buffer exchange in petri dishes can be achieved by turning the dish away from you and quickly removing buffer at the edge of the egg mass using a wide-bore Pasteur pipet prior to pouring in the next wash buffer. During the washes, the eggs should be "gardened," i.e., visibly deformed eggs, activated eggs, puffballs, and large pieces of debris should be removed using the wide-bore Pasteur pipets. However, one should not get so caught up in removing bad eggs and debris that this slows the preparation of

the extract. Time is another important variable. Generally it should be possible to go from collecting of eggs to the crushing spin in 45 min to 1 hr.

D. Procedure for Extract Preparation

1. Combine batches of good laid eggs and remove as much MMR as possible.
2. Wash eggs in 2 or 3 liter of MMR until all of the debris is removed. Combine good egg batches in a frog bucket, rinse a couple of times with MMR, and pour the eggs into a 600-ml beaker for the rest of the MMR washes. Since eggs settle quickly, pouring out the MMR immediately after the eggs have settled allows easy removal of debris. Eggs should be gardened during this step and during washes after dejellying.
3. Remove as much MMR as possible and rinse eggs into dejellying solution.
4. Swirl gently and intermittently in the dejellying solution. While the eggs are dejellying, pipet 1 ml of CSF-XB + PIs to each 13 × 51-mm Ultraclear centrifuge tube and add 10 μl of 10 mg/ml cytochalasin B/D per tube (flick the tube well immediately after pipeting in the cytochalasin or it will precipitate).
5. After eggs are dejellied (this will take 7–10 min and the volume will decrease approximately five-fold; eggs will pack tightly and orient with their vegetal poles down), remove as much dejellying solution as possible, rinse eggs with residual dejellying solution and once with XB, then transfer eggs to gelatin-coated petri dishes. Wash dejellied eggs three or four times with XB in the petri dish as described in Section II,C. For larger batches of eggs, we recommend using the high-sided 150-mm petri dishes.
6. Wash eggs two or three times in CSF-XB. Remove as much buffer as possible.
7. Wash eggs twice in CSF-XB + PIs. Leave eggs in a small volume of CSF-XB + PIs after the second wash.
8. Draw eggs up into the wide-bore Pasteur pipet and slowly drop them into the 1-ml CSF-XB + PIs + 100 μg/ml cytochalasin solution in the ultraclear tube. Insert the pipet below the meniscus to break the surface tension and gently release eggs into the solution. Minimize transfer of buffer along with the eggs.
9. Aspirate excess buffer from the top of the eggs.
10. Put the ultraclear tubes into 14-ml polypropylene round-bottom culture tubes (Falcon No. 2059) and spin for 10 sec at setting No. 4 (approx. 1500 rpm) in a clinical centrifuge.
11. Gently aspirate buffer from top of the eggs and layer on 0.75–1 ml of Versilube F-50. Use of versilube minimizes dilution during extract preparation. The density of versilube is intermediate between that of buffer and cytoplasm; thus, versiluble displaces buffer between the eggs during the packing spin but floats to the top of the cytoplasmic layer during the crushing spin.

12. Pack the eggs by spinning at setting No. 5 for 30 sec (2000 rpm) and full speed (No. 7) for 15 sec (2500–3000 rpm) in the clinical centrifuge.

13. Aspirate all buffer and versilube from the top of the packed eggs.

14. Transfer the tubes containing the packed eggs to the SW55.1 rotor in the ultracentrifuge. Crush the eggs by centrifugation at 10,000 rpm for 15 min at 16°C (full brake).

15. Store tubes with crushed eggs on ice. The light yellow layer on top represents the lipid droplets and the dark layer on the bottom contains the yolk and nuclei. The cytoplasmic layer is the muddy or straw-colored layer in the middle of the tube. Prior to collecting the cytoplasmic layer, wipe the sides of the tubes with 95% ethanol. Puncture the tube near the bottom of the cytoplasmic layer with an 18-gauge needle (on a 1-cc syringe), and gently draw out the extract. [See Fig. 3 of Murray (1991) for a picture of an extract.] We find it best to use a new needle for each centrifuge tube. Transfer the collected extract to a 5-ml snap cap tube and estimate its volume.

16. Add 1/1000 volume of protease inhibitor (LPC) and cytochalasin stocks, 1/20 volume of 20× energy mix, and 1/40 volume of 2 *M* sucrose. Note that some people do not add additional sucrose to the extract after preparation, but we find that it often helps stabilize the extract during immunodepletions (see Section V,A).

E. Preparation and Use of Sperm Nuclei and Fluorescent Tubulin

Sperm nuclei are prepared exactly as described by Murray (1991) and stored in small aliquots at −80°C at a density of 1–5 × 10^7/ml. Sperm nuclei can be frozen and thawed multiple times without apparent loss of activity, but we prefer to store them in small aliquots and only freeze–thaw once for spindle assembly reactions. Sperm nuclei are added to extracts at a 1/100–1/200 dilution yielding a final concentration of 100–300 nuclei/μl.

Fluorescent tubulin is used to monitor microtubule distributions during spindle assembly and is prepared by the high pH labeling protocol described by Hyman *et al.*, (1991). Fluorescein, tetramethyl rhodamine, or X-rhodamine-labeled tubulin can be used to monitor spindle assembly. We generally use X-rhodamine-labeled tubulin because it has better spectral separation from fluorescein than does tetramethyl rhodamine, allowing double-label immunofluorescence studies with fluorescein-conjugated secondary antibodies (see Section IV). Our X-rhodamine-labeled tubulin preps have final concentrations varying between 15 and 25 mg/ml with labeling stoichiometries between 0.5 and 1. For monitoring spindle assembly, we add X-rhodamine tubulin to the extract at a final concentration of 20–50 μg/ml (generally a 1/400 dilution). Although tubulin by itself is highly unstable, it appears to be very stable in the extract and can be added immediately after extract preparation.

III. Spindle Assembly Reactions

Three types of spindle assembly reactions have been described in the literature (Sawin and Mitchison, 1991; Heald *et al.,* 1996). In the first type of reaction, termed CSF spindle assembly, each sperm nucleus added to the extract drives the formation of a half spindle. Two such half spindles then fuse to form a bipolar mitotic spindle (Fig. 1, top) (Sawin and Mitchison, 1991). Thus, two haploid sperm nuclei drive the formation of a single bipolar spindle. In the second type of reaction, termed cycled spindle assembly or interphase-to-mitosis spindle assembly, calcium is added to a CSF extract containing sperm nuclei, inactivating the CSF arrest and driving the extract into interphase. As the nuclei in the extract cycle through interphase their DNA is replicated once (Sawin and Mitchison, 1991). Fresh CSF extract without any sperm nuclei is then added to drive the extract containing replicated sperm nuclei into metaphase. In this type of spindle assembly reaction, each sperm nucleus undergoes one round of replication and drives the formation of a bipolar mitotic spindle (Fig. 1, bottom). Cycled spindles containing replicated chromosomes are arrested in metaphase by CSF; addition of calcium for a second time results in inactivation of CSF and anaphase chromosome segregation (Shamu and Murray, 1992). Heald and coworkers (1996) described the assembly of spindles around DNA-coated beads in extracts. These studies demonstrated that chromatin-coated beads are sufficient to generate a bipolar mitotic spindle in the absence of centrosomes and kinetochores. A study describing the use of DNA-coated beads for studying spindle assembly has been presented recently (Heald *et al.,* 1998).

A. General Considerations for Spindle Assembly Reactions

Xenopus extracts are a powerful tool for studying mitosis but their use can be very frustrating because of high variability. These extracts are very sensitive to physical perturbations and must be treated gently to ensure the best results. Avoid pipeting up and down vigorously with narrow-bore pipet tips or dropping tubes containing extract reactions since either of these can sufficiently perturb the extract to disrupt the spindle assembly process. We recommend pipeting with either commercially available wide-bore P-200 tips (Rainin HR-250W) or regular P-200 tips that have been cut off to an opening of ~2 mm diameter and mixing by either gently tapping the bottom of the tube or inverting the tube two or three times. Use a fresh pipet tip for each aliquot, especially for experiments requiring quantitation because these extracts are very viscous. Never vortex a tube containing extract because this will surely destroy the extract. Finally, avoid diluting the extract with buffer or other reagents because this can also inhibit spindle assembly (see Section V,B).

To set up spindle assembly reactions, add sperm nuclei and labeled tubulin to a large volume of extract and then aliquot it into individual reactions. The extract is very viscous and difficult to pipet accurately. Use a fresh pipet tip for

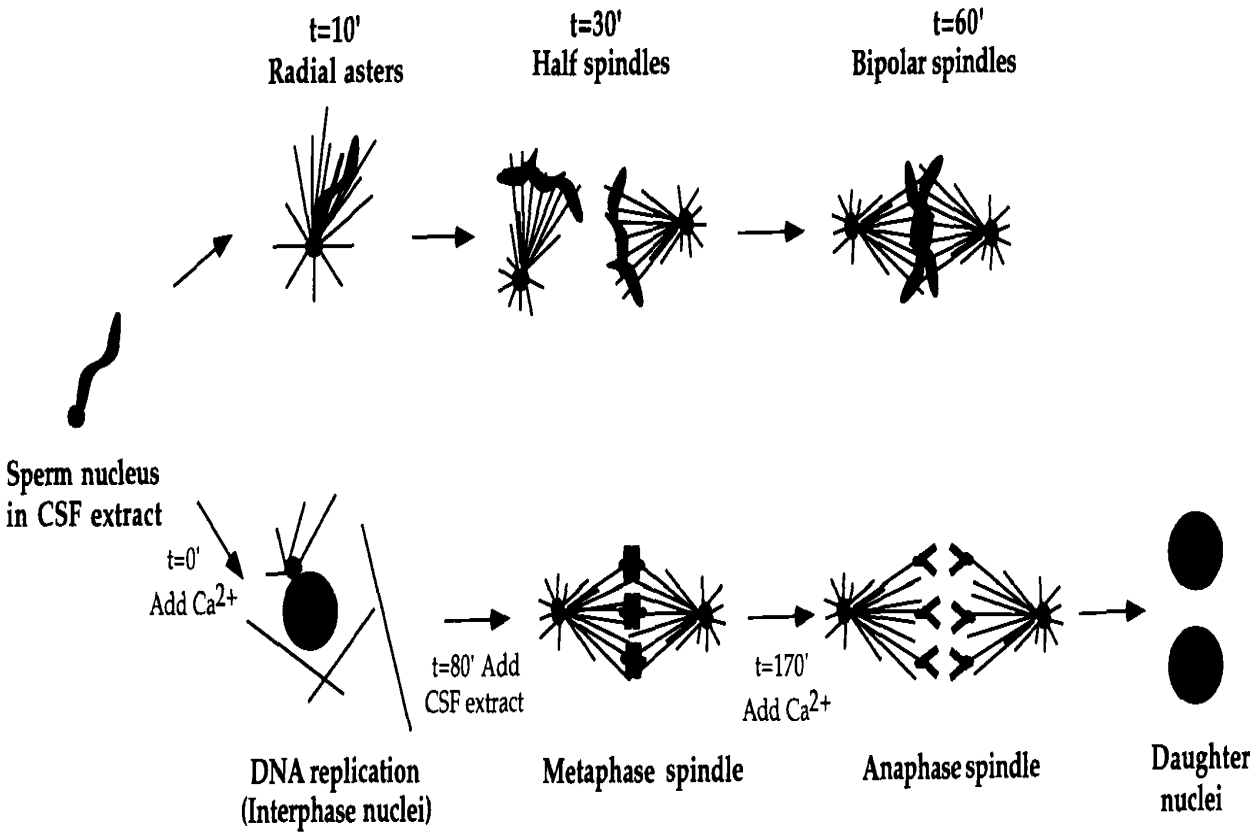

Fig. 1 The *in vitro* spindle assembly pathways. (Top) CSF spindle assembly. (Bottom) Cycled spindle assembly.

each aliquot to ensure reproducibility and allow for a 20% volume loss upon aliquoting. Do not exceed a reaction volume of >50 μl/1.5 ml Eppendorf tube since larger reaction volumes tend to inhibit spindle assembly. We recommend incubating reactions at 20°C in a cooled water bath (a home-made one can be a plastic tray containing water with some ice to maintain 20°C). However, if the room temperature is stable from day to day and does not exceed 24 or 25°C, then the reactions can be incubated on the bench top.

B. Reagents Required for Spindle Assembly

Sperm nuclei at 1–5 × 10^7/ml

Fluorescently labeled tubulin

Extract fix: 60% (v/v) glycerol, 1× MMR, 1 μg/ml Hoechst 33342/33258, 10% formaldehyde [from 37% (w/v) stock]

Sperm dilution buffer (1×): 10 m*M* Hepes (pH 7.7), 1 m*M* $MgCl_2$, 100 m*M* KCl, 150 m*M* sucrose, 10 μg/ml cytochalasin B/D; can be prepared as a 5× stock; both 1× buffer and 5× stock should be stored at −20°C

10× calcium: 4 m*M* $CaCl_2$ in 1× sperm dilution buffer

C. CSF Spindle Assembly

To CSF extract containing labeled tubulin add sperm nuclei to a final concentration of 100–300/μl. Mix gently, aliquot 25 μl/tube, and incubate at 20°C. At 15, 30, 45, and 60 min take samples to test the progress of the reaction as follows: pipet a 1-μl aliquot of the reaction on a microscope slide, overlay with 3 μl of extract fix, cover gently with an 18 × 18-mm coverslip, and view by fluorescence microscopy. At the 15-min time point, small microtubule asters emanate from the sperm centrosome (Fig. 2, top); these often have a very dense core of microtubules. By 30 min the chromatin has migrated away from the centrosome, and the microtubules are polarized toward the chromatin: These structures are termed half spindles (Fig. 2, middle). Between 30 and 60 min, half spindles begin to fuse to form bipolar spindles (Fig. 2, bottom). During CSF spindle assembly, the percentage of total structures that are bipolar spindles varies considerably from extract to extract. In a typical extract, 40–60% of the total structures are bipolar spindles by the 60-min time point. This number can range from <10% in very poor extracts to >90% in the best extracts.

D. Cycled Spindle Assembly

For cycled spindle assembly the extract is divided into two tubes after the addition of labeled tubulin. One tube is supplemented with sperm nuclei and cycled into interphase by the addition of calcium, whereas the second tube is held on ice until it is needed to drive the extract with replicated sperm nu-

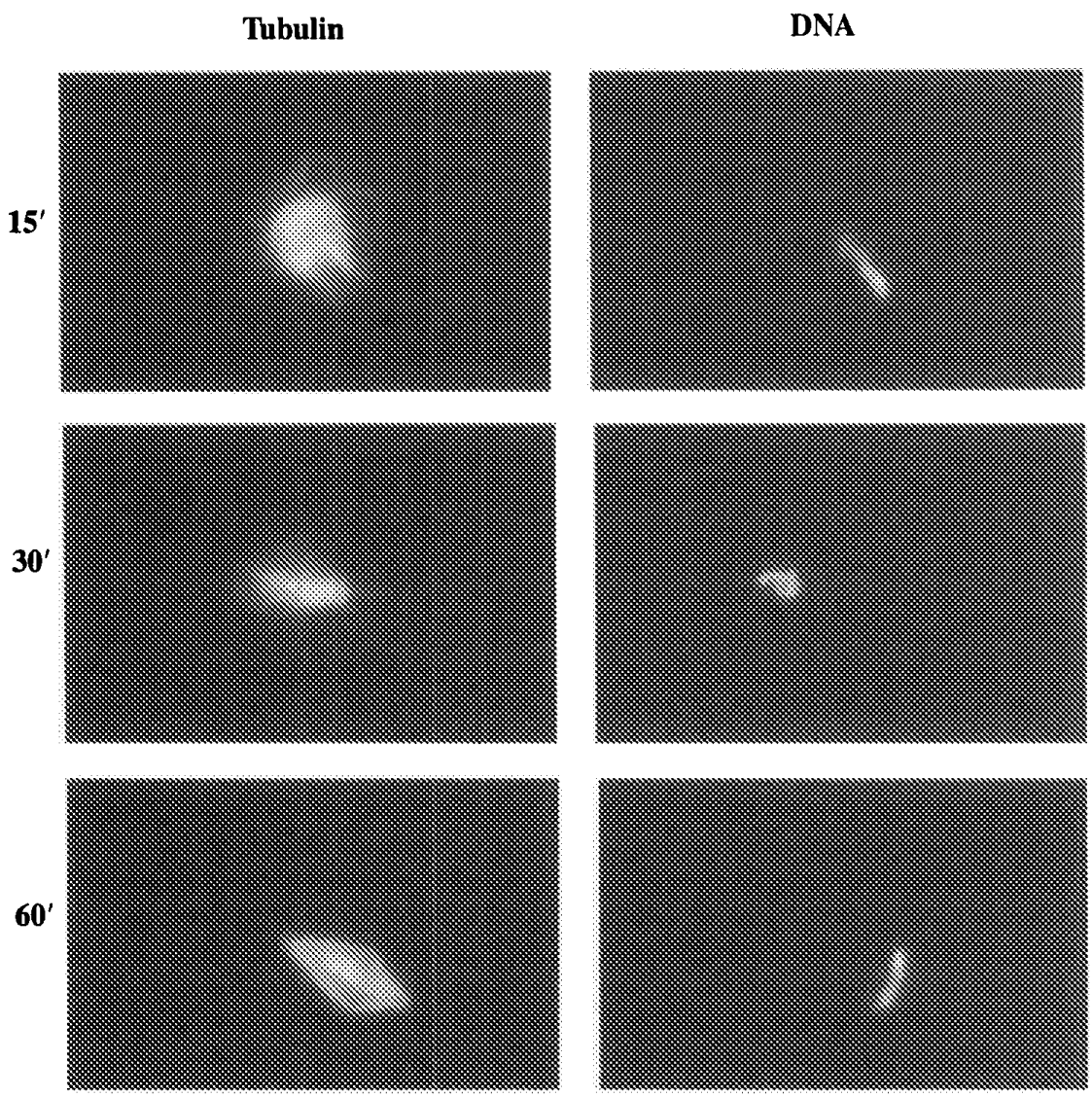

Fig. 2 Reaction intermediates of CSF spindle assembly reaction. Samples were removed from the reaction and sedimented onto coverslips as described in the text. The microtubules and chromatin structures formed during the reaction are shown for 15-, 30-, and 60-min time points.

clei back into metaphase. Specifically, to one-half of the extract containing labeled tubulin add sperm nuclei. Withdraw a small amount as a negative control (~20 μl), and to the rest add 1/10 volume of 10× calcium solution. Mix the calcium into the extract gently but thoroughly, aliquot 20-μl/tube, and incubate both the aliquot withdrawn prior to calcium addition and the reaction tubes supplemented with calcium at 20°C for 80 min. During this incubation the extract is monitored by taking samples at 30-, 45-, and 60-min time points as described in Section III,C. Reactions to which calcium was added should exit the CSF arrest and cycle through interphase; negative control sample (withdrawn before calcium addition) should remain in CSF (mitotic) arrest. Morphology of the

mitotic arrest was described in Section III,C. The reactions with calcium should appear as follows: after 30 min, the sperm nucleus should be swollen and many free microtubules should be present in the extract; after 45 min, the sperm nucleus should be large and round and resemble a typical mammalian cell interphase nucleus; after 60 min, the sperm nucleus should appear large and reticular much like an early prophase nucleus, suggesting that the extract may be starting to cycle back into metaphase. In our experience, extracts that have irregularly shaped interphase nuclei at the 45-min time point tend to be less robust in generating mitotic spindles. After 80 min at RT, 20 μl of CSF extract containing labeled tubulin but not sperm nuclei is added to each reaction (this represents an equal volume of extract added to the initial reaction), mixed in gently and incubated at 20°C for 60–90 min to allow establishment of the metaphase CSF arrest and formation of bipolar spindles. The reactions containing bipolar spindles can be held at RT for many hours without detriment, although with increased time the number of single bipolar spindles drops significantly because the spindles tend to aggregate laterally, forming large multipolar structures.

In general, cycled spindle assembly results in a more uniform distribution of structures than CSF spindle assembly and yields a higher proportion of bipolar spindles (80–90% of total structures at 60 min). Cycled spindles have chromosomes aligned in an equatorial plane, highly reminiscent of a metaphase plate in somatic cells (Fig. 3, top). However, the tightness of the metaphase plate in cycled spindles can vary significantly between extracts. In good extracts, the chromosomes are tightly focused at the spindle equator, whereas in less robust extracts, the chromosomes tend to be both at the spindle equator and scattered on either side of the equator. These less robust extracts generally do not work well for anaphase chromosome segregation (see Section VII).

The morphological pathway by which cycled spindles assemble remains to be clarified. Sawin and Mitchison (1991) first described the pathway as being chromatin driven, i.e., microtubules assembled in the region of the chromatin and with time organized into a bipolar structure with morphologically distinguishable poles. This pathway is similar to that described recently using chromatin-coated magnetic beads, with the exception that spindles assembled from sperm nuclei contain centrosomes. However, Boleti and coworkers (1996) reported that two distinct centrosomal arrays of microtubules are separated to opposite sides of the nucleus in a more classical somatic cell-type spindle assembly pathway. A clear picture of the cycled spindle assembly pathway awaits a thorough real-time study of this reaction using fluorescence video microscopy.

IV. Monitoring Spindle Assembly Reactions

A. Methods for Pelleting Spindles onto Coverslips

Spindle assembly reactions are routinely monitored using fixed squashes as described in Section III,C. However, for quantitative analysis we prefer to sedi-

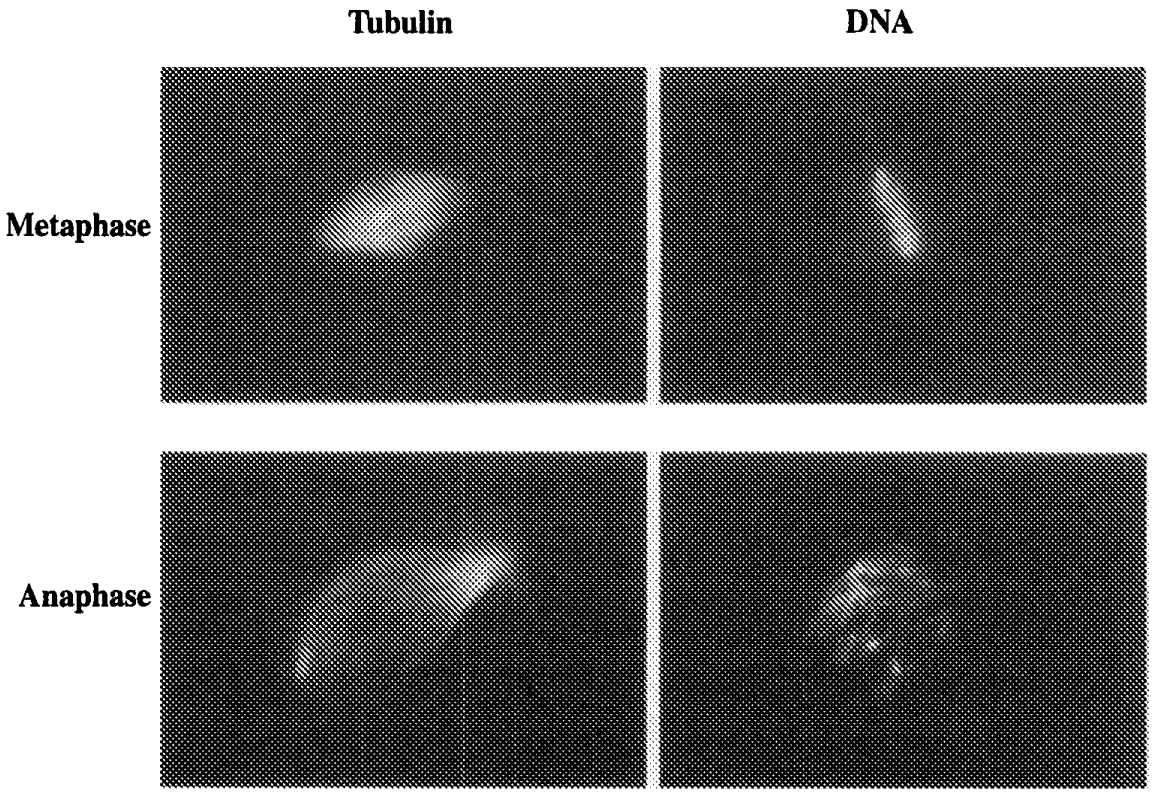

Fig. 3 Optimal fixation of spindle structures. Using the optimized fixation conditions it is possible to preserve chromosome structure and microtubule structure. (Top) Metaphase spindles assembled by the cycled spindle pathway. (Bottom) Spindles that have been induced to enter anaphase following calcium addition.

ment the spindles from a large volume (20 μl) of extract onto a coverslip. The advantages of sedimenting spindles onto coverslips are threefold. First, spindles from 20 μl of reaction are concentrated onto a 12-mm coverslip, providing a dense, relatively homogenous sample for quantitative analysis. Second, having the spindles fixed on coverslips allows one to perform immunofluorescence to analyze the localization of specific antigens on the *in vitro* spindles. Third, unlike squashes, the pelleted samples are very stable when stored at −20°C. This allows one to prepare many samples at once but analyze them when convenient and store them for any future reanalysis. We use two procedures for pelleting spindles onto coverslips. In the first (method 1), reactions are diluted extensively into a microtubule-stabilizing buffer, pelleted unfixed onto coverslips, and fixed onto the coverslips using methanol. Two limitations of this method are an extreme disruption of chromosome structure and day-to-day variability. Recently, we have developed an alternative method (method 2) that involves first diluting the spindles into a microtubule-stabilizing buffer and then fixing the spindles in solution using formaldehyde, pelleting the spindles onto coverslips, and postfixing using methanol. Using method 2 we have satisfied the demanding requirement

of retaining the fine structure of chromosomes as they are undergoing anaphase without causing significant disruption of spindle structure (Fig. 3, bottom).

1. Requirements for Pelleting Spindles onto Coverslips

BRB80
80 m*M* K-Pipes, pH 6.8
1 m*M* $MgCl_2$
1 m*M* EGTA
Make as 5X stock, sterile filter and store at 4°C

5-ml snap cap tubes (Sarstedt No. 55.526.006)

Dilution buffer
BRB-80
+ 30% (v/v) glycerol
+ 0.5% Triton X-100

Fixation buffer
BRB80
+ 30% (v/v) glycerol
+ 0.5% Triton X-100
+ 4% formaldehyde, added just before use from 37% stock

Cushion
BRB80
+ 40% (v/v) glycerol

Spindown tubes (Evans *et al.*, 1985)
With 12-mm coverslips and 4-ml cushion at room temperature

Coverslip holders
For 12-mm round coverslips; we like to use Thomas Scientific No. 8542-E40

−20°C methanol

TBS-TX
1X TBS [10 m*M* Tris (pH 7.4) and 150 m*M* NaCl]
0.1% Triton X-100

Mounting medium
0.5% *p*-phenelynediamine (free base) in 90% glycerol and 20 m*M* Tris–Cl, pH 9.0

2. Method 1

1. Prior to beginning the spindown procedure, prepare spindown tubes with coverslips and cushion and 5-ml snap cap tubes with 2 ml of dilution buffer. All buffers should be at RT.

2. Pipet 20 μl of spindle assembly reaction into the dilution buffer. Prior to transferring the extract into the dilution buffer, we often pipet up and down two or three times with a regular yellow tip to break any large aggregates.

3. Immediately cap the tube and mix by gently inverting four or five times.

4. Using a disposable plastic transfer pipet, layer the diluted sample onto the cushion in the spindown tube. After dilution, the spindles are stable; therefore, if a large number of samples are being pelleted, first do the dilution and mixing for all samples and then layer them sequentially onto cushions.

5. Centrifuge the tubes in an HS-4 rotor at 18°C for 20 min at 5500 rpm (6000*g*). We use the HS-4 because it can hold 16 tubes; an HB-4, HB-6, or equivalent rotor can be used for smaller numbers of tubes.

6. Aspirate until just below the sample–cushion interface, rinse with BRB80, aspirate off the rinse and the cushion, and transfer the coverslips to a coverslip holder.

7. After all coverslips are in the holder, fix by immersion in −20°C methanol for 3 min.

8. Rehydrate using two sequential 5-min incubations in TBS-TX.

9. Label DNA by rinsing coverslips with 1 μg/ml Hoechst in TBS-TX for 30 sec, mount in mounting medium, and seal with nail polish. Rinse top surface of coverslip with water prior to observation.

3. Method 2

Method 2 is different from method 1 as follows:

1. Dilute 20 μl of sample into 1 ml of dilution buffer as in step 2 of method 1.

2. Dilute all samples first, then add 1 ml of fixation buffer and mix well by inversion.

3. Fix for 5 min at RT prior to layering onto cushion.

4. After pelleting fixed spindles onto coverslips and aspirating the cushion after rinsing (step 6 in method 2) postfix the coverslips in −20°C methanol.

Using this method we have successfully preserved the fine structure of chromosomes (Fig. 3) without significantly compromising spindle structure.

Spindles pelleted onto coverslips using either method can be processed for immunofluorescence using antibodies to specific spindle components (Walczak *et al.*, 1996). After rehydrating in TBS-TX, coverslips are blocked in TBS-TX + 2% BSA (antibody dilution buffer or AbDil) for 15–30 min, incubated sequentially with primary and secondary antibodies diluted in AbDil, rinsed in TBS-TX containing 1 μg/ml Hoechst, and mounted as described previously. For localizing antibodies added to inhibit function of a particular component, only incubation with the appropriate secondary is necessary (Walczak *et al.*, 1997).

B. Time-Course Experiments

While characterizing the functions of spindle components using the CSF spindle assembly reaction, it is often desirable to monitor the intermediates in the path-

way. In order to set up time-course experiments for this purpose, it is important that samples for all time points are derived from a single pool of reaction mix and that they are sedimented onto coverslips at the same time. Specifically, to a volume of extract sufficient for all time points, add sperm nuclei and any other reagents, such as antibodies or dominant-negative fusion proteins, and store on ice. At various intervals beginning with $t = 0$ min, remove 25 μl and transfer to a tube at 20°C. At the end of the time course, pellet all samples onto coverslips using either of the methods described in Section IV,A.

V. Manipulation of Extracts

The ability to manipulate specific components in the extract by either immunodepletion or reagent addition and to assay the effects of these manipulations on spindle assembly is one of the main advantages of *Xenopus* egg extracts. A recent flurry of publications applying this strategy to dissect the role of both motor and nonmotor components during spindle assembly demonstrates the value of this approach (Sawin *et al.*, 1992; Vernos *et al.*, 1995; Boleti *et al.*, 1996; Heald *et al.*, 1996, 1997; Merdes *et al.*, 1996; Walczak *et al.*, 1996, 1997).

A. Immunodepletion of Extracts

The main difficulty with immunodepletion of CSF extracts is that they lose the CSF arrest during or soon after immunodepletion. In our experience, the following actions seem to aid in immunodepleting proteins without losing the CSF arrest:

1. During the CSF extract prep, after washing with XB, the eggs are washed thoroughly with CSF-XB without protease inhibitors (step 6 in Section II,D). This CSF-XB wash ensures that there is sufficient EGTA in the final extract to maintain a tight CSF arrest.

2. Eggs are crushed at 10,000 rpm (full brake) in a SW55 rotor at 16°C. Even a slightly faster spin (12,500 rpm) does not work well—one does not obtain robust spindle assembly after immunodepletion.

3. Antibodies are coated on BioRad Affi-Prep Protein A beads (BR No. 156-0006). Unlike regular protein A agarose beads, these high-density beads sediment readily through the viscous extract at speeds gentle enough to not perturb the extract.

4. Remove as much buffer as possible after coating the beads with antibody to avoid diluting the extract, which will inhibit spindle assembly.

5. Extracts are handled extremely gently during manipulations such as resuspending beads or sedimenting beads.

The ability to deplete a specific protein from the extract is dependent on both the quality of the antibody used for depletion and the abundance of the protein

being depleted. Regarding antibody quality, for reasons we do not understand, we have had much better success with antibodies prepared by immunizing with native proteins as opposed to antibodies generated against denatured proteins, e.g., proteins excised from acrylamide gels.

The following protocol is for depleting a protein present at ~10–20 μg/ml in the extract using high-affinity polyclonal antibodies. While this presents a good starting point, exact conditions must be optimized for each new antibody, particularly the amount of antibody needed for maximal depletion. As a control, we use an equivalent amount of random IgG from the same species; given the extreme perturbation of the extract by the immunodepletion procedure all interpretations of depletion phenotypes must be restricted to comparison with random IgG depletion performed at the same time and processed identically. The procedure for depletion is as follows:

1. Pipet 25 μl of Affi-Prep protein A beads (50 μl of slurry) into a 0.5-ml Eppendorf tube.
2. Wash beads three times with 0.5 ml TBS-TX [20 m*M* Tris (pH 7.4), 150 m*M* NaCl, and 0.1% Triton-X 100].
3. Add 4 μg of antibody and bring total volume to 200 μl using TBS-TX.
4. Bind antibody to beads at 4°C for 1 hr on rotator. Make sure beads are rolling around during the incubation.
5. Pellet beads in a microfuge for 20 sec, wash one time with TBS-TX and three times with CSFXB + PIs using 200 μl/wash. Remove as much buffer as possible to avoid diluting the extract.
6. Add 200 μl of extract to each tube and resuspend beads in extract very gently using a wide-bore P-200 tip; Do not pipet up and down more than three times and avoid tapping the tube or any other type of vigorous agitation.
7. Before the beads settle, rapidly place the tube on the rotator and rotate for 1 hr at 4°C. Make sure that the beads are mixing well with the extract during this period.
8. Pellet the beads for 20 sec in a microfuge and transfer the supernatant to a fresh tube—this is the depleted extract. It is difficult to recover more than 175 μl from a 200-μl depletion without contaminating the extract with the beads. Save a small amount of depleted extract to test the extent of depletion by immunoblotting.
9. To analyze what is bound to the beads, wash the beads two times with CSF-XB + PIs, three times with TBS-TX, and one time with TBS. Resuspend the beads in 50 μl SDS–PAGE sample buffer and boil to release the IgG–antigen complexes from the beads. Analyze the sample by SDS–PAGE and Coomassie brilliant blue staining.

Extracts that have been immunodepleted are far less robust than nondepleted extracts. A good CSF extract will remain mitotic for at least 6–10 hr on ice,

whereas a depleted extract will remain mitotic for a few hours at most. We find that with depleted extracts CSF spindle assembly proceeds normally, although the extent of bipolar spindle formation is reduced by twofold compared to the undepleted extract (Walczak *et al.*, 1997). At a frequency lower than that for CSF spindle assembly, we have successfully used immunodepleted extracts for cycled spindle assembly. We have yet to attempt anaphase chromosome segregation in depleted extracts.

We have tried to optimize the immunodepletion procedure to minimize perturbation of the extract but have yet to find the perfect conditions. We believe that the primary problem with immunodepletions is the physical perturbation of the extract by the beads as they are sloshing through the extract. Our most successful manipulation to minimize extract perturbation has been to reduce the time of depletion to 30–45 min instead of 1 hr. However, with lowered times we also see a reduced efficiency of depletion; we have yet to systematically investigate this issue. We have also wondered whether doing the depletions at RT might be better since calcium uptake machinery would sequester released calcium more efficiently at RT than at 4°C and also whether adding antibody directly to extracts and collecting the antibody–antigen complexes later might be faster and less disruptive than using antibody-coated beads. Addition of excess EGTA to sequester released calcium never gave reproducible improvements. Given the logical importance of immunodepletion experiments, technical improvements to immunodepletion procedures are eagerly awaited by many researchers.

B. Reagent Addition to Extracts

A complementary approach to immunodepletions is to assay the effect of adding either inhibitory antibodies or dominant-negative proteins on spindle assembly. This approach is useful because it is less perturbing to the extract than immunodepletion. In addition, it allows one to perturb protein function both before and after spindle assembly, thus allowing one to address the requirement of protein function in both the establishment and maintenance of spindle structure.

One concern with respect to addition of reagents to extracts is dilution of the extract. We recommend not adding reagents to >10% of extract volume since greater dilution results in poor extract performance. For antibodies, we recommend dialyzing into a buffer that is compatible with extracts, such as 10 m*M* Hepes (pH 7.2) and 100 m*M* KCl. In this buffer, several antibodies that we generated are stable at −80°C and working stocks are stable for several months at 4°C. Also compatible with extracts is 50 m*M* K-glutamate (pH 7.0) and 0.5 m*M* $MgCl_2$, and this can also be used for storage of antibodies. We avoid the use of CSF-XB or sperm dilution buffer for storing antibodies since both these buffers contain sucrose, making them highly susceptible to bacterial contamination at 4°C. We also avoid adding azide to our antibody stocks. Dominant-

negative fusion proteins can be dialyzed into CSF-XB and stored at −80°C prior to addition to extracts.

The amount of antibody that must be added to perturb function depends on the quality of the antibody being used and on the abundance of the target protein in the extract. We have found that as little as 25–50 μg/ml of a very potent antibody, such as the polyclonal anti-XKCM1 antibody, completely inhibits XKCM1 function, whereas 1 mg/ml of a monoclonal antibody to the intermediate chain of dynein is required to inhibit dynein function. We suggest that each new antibody be titrated to determine the amount necessary for full inhibition. Since the mode of action of dominant-negative proteins is presumably to compete with the endogenous protein, the amount of fusion protein that must be added to perturb function depends on the endogenous concentration of the target protein. We recommend a 10-fold molar excess of fusion protein as a starting point but the concentration for optimal inhibition must be empirically determined for each protein.

VI. Data Analysis and Interpretation

Xenopus extracts are very powerful for analyzing protein function in spindle assembly, but all extract workers admit that their use is plagued by variability. It is not uncommon to go through periods in which several concurrent extracts will not assemble good spindles or in which immunodepletions consistently lead to extract activation. This is perfectly normal and can only be overcome by persistence. However, the extreme variability does necessitate care in performing and interpreting experiments, and we discuss how we approach the analysis of protein function in spindle assembly.

To analyze spindle assembly defects, we recommend starting with technically simpler antibody addition experiments, varying the concentration of added antibody and analyzing the effect using spindowns to facilitate detailed analysis. We quantitatively characterize the phenotype in at least three different experiments on three different extracts, often preparing duplicate samples on a single extract to increase the sample size. Once the effect of antibody addition is clear, the next step is to determine the effect of protein depletion. The immunodepletion procedure itself perturbs the extract significantly, making it difficult to discern subtle phenotypes. Furthermore, immunodepletion is technically much more difficult to execute than antibody addition. However, the results of antibody addition experiments must be interpreted cautiously in the absence of a confirmation of the observed effects by immunodepletion. In the absence of immunodepletions, combining antibody inhibition with a dominant-negative approach might help strengthen conclusions with respect to the role of a specific protein in spindle assembly. In addition, determining the effect of manipulating a particular protein on all three types of spindle assembly reactions—CSF spindle assembly, cycled spindle assembly, and DNA bead spindle assembly—can also provide greater

insight into its role in spindle assembly (Heald *et al.*, 1997). Performing identical manipulations on different types of spindle assembly reactions can also provide new insights into the process of spindle assembly itself, as demonstrated by recent work characterizing the role of centrosomes during spindle assembly (Heald *et al.*, 1997). Finally, the most desirable extension of the manipulation of specific components during spindle assembly is to reconstitute spindle assembly by adding back purified protein to immunodepleted extract—the logical equivalent of a genetic complementation. However, as might be expected, this has proven to be quite difficult and has only been partially achieved (Merdes *et al.*, 1996; Walczak *et al.*, 1996). Future technical improvements in immunodepletion procedures will be necessary to achieve this goal.

The extreme variability between extracts makes identifying the effect of manipulating specific components heavily dependent on the strength of the observed phenotype. Certain phenotypes, such as the one induced by inhibition of XKCM1, are extremely dramatic and can be easily defined in the first extract analyzed (Walczak *et al.*, 1996). Others, such as the one induced by inhibition of the spindle motor XCTK2, are much more subtle and difficult to detect without careful inspection of many extracts (Walczak *et al.*, 1997). Reproducibility and quantitative analysis become critical in determining the nature of subtler phenotypes.

VII. Anaphase *in Vitro*

Cycled spindles assembled in *Xenopus* egg extracts are capable of undergoing anaphase chromosome segregation (Shamu and Murray, 1992). This *in vitro* anaphase reaction has proven valuable for dissecting both the mechanism of sister chromatid separation and the mechanism of chromosome movement (Murray *et al.*, 1996; Desai *et al.*, 1998). However, successful anaphase *in vitro* requires considerably higher quality extracts than those used for spindle assembly and this has significantly limited the use of this reaction. In the following sections we provide some hints to facilitate successful preparation of anaphase-competent extracts. We also describe methods to set up and monitor an anaphase reaction using either fixed time points or time-lapse fluorescence microscopy.

A. Preparation of Anaphase-Competent Extracts

Extracts for monitoring anaphase are prepared exactly as described for CSF extracts (see Section II). Anaphase extracts require high-quality eggs, and frogs which lay even small amounts of puffballs or stringy eggs should not be used. Note that the absolute volume of extract required for these experiments is very small and quality should always take precedence over quantity. One factor, which should be minimized in the preparation of anaphase-competent extracts, is the time between egg-laying and extract preparation. Often the best extracts are

prepared from freshly squeezed eggs since they have not been floating for several hours in "frog-conditioned" MMR. However, laid egg extracts, which are easier to prepare, can also be fully competent for anaphase. We routinely collect laid eggs from four to six frogs about 12–14 hr after injection with hCG (frogs are stored at 16°C in 1X MMR as described previously, generally two to four of the frogs lay eggs of sufficient quality for extract preparation) and prepare extracts from the laid eggs and freshly squeezed eggs in parallel. While squeezing the frogs, it is extremely important to avoid getting any frog skin secretions ("frog slime") into the squeezed eggs or the eggs will activate. Frog slime can be avoided by frequently dipping the frog into a bucket of clean distilled water during the squeezing process. Squeezed eggs from different frogs should be kept separate initially but can be pooled after dejellying if they do not exhibit signs of activation. It is difficult to obtain large quantities of squeezed eggs, but from four frogs we generally get enough to fill one-half to three-fourths of a 13 × 51-mm Ultraclear tube. The laid eggs and squeezed eggs are separately processed as described previously for the CSF extract prep. It is important to thoroughly wash out residual jelly coat after the dejellying step and to crush the eggs at 10,000 rpm for 15 min at 16°C (full brake). This speed spin results in highly turbid muddy-colored extracts. Faster spins result in clearer extracts that often suffer from extensive and rapid spindle aggregation, resulting in very limited time of manipulation and precluding any significant analysis. We also note that newer frogs that have not been through multiple rounds of ovulation tend to be better for anaphase experiments, although, as with many other aspects of frog egg extracts, this is by no means definitive. Using frogs that have been through less than two cycles of ovulation (with at least a 3-month rest between ovulations) after being obtained from a distributor and taking great care in preparing the extracts, we have been able to successfully obtain anaphase extracts on a routine basis (70% of the time).

B. Setting Up and Monitoring Anaphase Reactions

Anaphase reactions require spindles obtained by cycled spindle assembly as described in Section III,D. It is essential that the cycled spindles have tight metaphase plates; often, chromosomes on the spindles are not confined to a tight metaphase plate but stray over the entire spindle, and these extracts are not useful for anaphase. To perform an anaphase reaction, 9 μl of a cycled spindle assembly reaction is mixed with 1 μl of the 10X calcium stock (see Section III,B) and monitored by taking fixed time points every 5 min for 30 min. The added calcium should be mixed well with the extract, either by gently flicking the bottom of the tube or by gently pipeting up and down. The addition of calcium results in the destruction of CSF and exit from the metaphase arrest. Between 5 and 15 min after calcium addition, clear evidence for anaphase should be evident in the chromosome morphology as depicted in Fig. 3. In addition to anaphase chromosome movement, changes in spindle structure, particularly thinning out of spindle MT density and expulsion of asters, should be evident (Murray *et al.*, 1996). By

25–30 min, rounded interphase nuclei should be visible in the reaction. In a good anaphase extract, 40–80% of the spindles show clear evidence of anaphase. However, it is not unusual to have an extract whose spindles have tight metaphase plates but do not exhibit anaphase. In addition, the percentage of spindles that exhibit anaphase often decreases dramatically with aging of the extract.

C. Real-Time Analysis of Anaphase *in Vitro*

1. Lengthening Extract Life Span

Although technically challenging, it is possible to observe anaphase *in vitro* in real time allowing detailed analysis of chromosome movement and spindle dynamics (Murray *et al.*, 1996; Desai *et al.*, 1998). Since there is no reliable procedure for freezing extracts while maintaining anaphase competency, and since each anaphase reaction takes approximately 30 min, during which only one spindle can be observed per field of view (of which <50% will provide analyzable data), it is essential to maximize the life span of the extract to perform real-time analysis. We have found it best to stagger the entire spindle assembly reaction by 3.5–4 hr, storing the original extract on ice during the interim, to maximize the amount of time that observational studies can be performed. We find this to be better than storing the extract on ice after cycling through interphase and have been able to use extracts routinely for 12–16 hr after preparation. We have also had some success with storing the cycled spindles at 10–12°C and warming them up to room temperature for 10–15 min prior to their use. We note, however, that with increased time spindles will be present in larger aggregates and the percentage of spindles capable of undergoing anaphase will decline.

2. Slide and Coverslip Cleaning

Requirements

Water, acetone, ethanol, slides, and coverslips (22 × 22 mm; No. 1), hotplate, slide storage box, 100-mm tissue culture dish, and coverslip spinner (optional).

It is essential to obtain homogenous sample films to avoid problems derived from air bubbles—we have often watched a spindle just beginning or in the middle of anaphase, only to have a bubble roll into view and wipe it out of existence. The key to good sample films is clean particulate-free slide and coverslip surfaces.

Cleaning Slides

1. Set up three 50-ml conical tubes filled with water, acetone, and ethanol and have a hotplate with a clean surface set such that the top feels hot to the touch (50–60°C).

2. Holding the frosted end, dip the slide several times in water and then in acetone and finally in ethanol. Transfer to the hotplate surface to rapidly dry

off the ethanol and store in a covered slide box. It is best to use a reasonably new box of slides to minimize particulates on the slides. If problems with bubbles persist, clean slides in a cup sonicator filled with warm water containing a small amount of detergent and rinse off the detergent prior to cleaning with acetone and ethanol.

Cleaning Coverslips

Coverslips are cleaned exactly as slides, except that coverslip holders are used for rinsing 12 coverslips at a time and the coverslips are dried using a homemade coverslip spinner. If no spinner is available, the coverslips can be dried by gently wiping both surfaces using lens paper (wear gloves while doing this). Clean coverslips can be stored in a plastic 100-mm tissue culture dish for several weeks.

3. Sample Preparation

Requirements

Valap, hotplate (for melting Valap), clean slides and coverslips, 10X calcium/DAPI stock (4 m*M* calcium chloride, 750 ng/ml DAPI in 1X sperm dilution buffer), extract reactions with cycled spindles.

Spindle microtubules are visualized using X-rhodamine tubulin added to the extract just after preparation. Chromosomes are visualized using DAPI and it is most convenient (and least perturbing) to add the DAPI to the calcium stock used for triggering anaphase. The 10X calcium/DAPI stock can be stored indefinitely at −20°C and is not sensitive to freezing and thawing. Prior to sample preparation, make sure that melted Valap is available to seal the sample. Valap is a 1:1:1 mixture of vaseline:lanolin:paraffin prepared by weighing equal amounts of the three components into a beaker and melting and mixing them on a hotplate.

Preparation of Sample

1. Transfer 9 μl of the cycled spindle assembly reaction to an Eppendorf tube.
2. Add 1 μl of the 10X calcium/DAPI stock. Mix gently but thoroughly by flicking and pipeting up and down.
3. Pipet 8 μl onto a clean slide. Cover gently with a clean 22 × 22-mm No. 1 coverslip. It is essential not to squash the sample film too vigorously. If there are many bubbles in the film, the sample should be discarded and a new sample should be prepared; however, it is often impossible to avoid one or two small bubbles near the center of the coverslip.
4. Seal the edges with Valap and start observation (a Q-tip works well as a Valap applicator).

We have experimented with more elaborate schemes to prevent distortion of spindles during sample preparation, such as coating of the slide surface with spacer latex beads, and also with alternative means of sealing the sample that avoid any local heating problems associated with the use of Valap (Murray *et*

al., 1996). However, we find that the simple procedure outlined previously is sufficient for observing anaphase and considerably reduces the complexity of sample preparation.

4. Sample Observation

Given the variable composition of products in a spindle assembly reaction, it is essential to pick the correct type of spindle for observation. We find it best to scan the sample in the DAPI channel using a 20× dry objective lens (with a 25% transmission neutral density filter in the light path) and to select medium-sized spindles with tight metaphase plates for time lapsing. Often, small spindles are too sensitive to flows under the coverslip (which are highly variable from extract to extract) that cause them to either spin around or float out of the field of view (both of which make analysis nearly impossible). Larger aggregates, while they do exhibit good anaphase, tend to be very messy and difficult to analyze. With a little practice, one can begin to pick spindles good for time lapsing anaphase with a frequency of 60–80%.

For acquiring images, we use low-power dry objectives (20×, 0.5 or 0.75 NA) and a cooled charged-coupled device camera. The minimal additional requirements for the real-time observation are the ability to acquire images at two different wavelengths (X-rhodamine and DAPI), to store the acquired images, and to shutter the illuminating light to minimize photodamage. A detailed description of the microscope setup used to acquire the images shown in Fig. 4 has

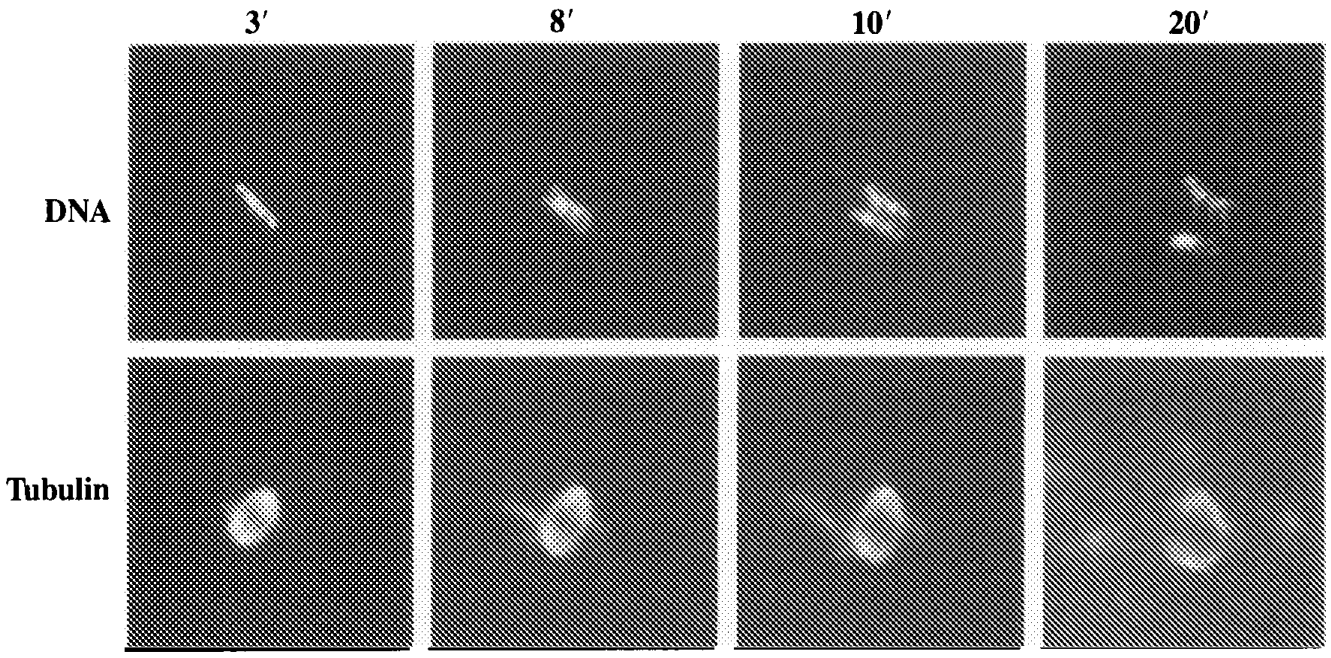

Fig. 4 Time course of anaphase chromosome movement. Individual frames are presented from a time-lapse recording of anaphase *in vitro*.

been published (Salmon *et al.*, 1994). Analysis of chromosome movement and spindle microtubule density can be performed on the collected images using one of several commercially available image analysis software packages.

D. Manipulation of Anaphase *in Vitro*

Manipulation of anaphase reactions has been restricted to addition of either nondegradable cyclin (cyclin Δ90) or pharmacological agents. For addition of cyclin Δ90, titrate the cyclin concentration such that physiological levels of H1 kinase are attained (Murray *et al.*, 1989). For optimal results, the cyclin Δ90 must be added to the extract for 20–30 min prior to triggering anaphase by addition of calcium. In the presence of cyclin Δ90 sister separation appears normal, although spindle microtubule density near the spindle poles does not decline and chromosome decondensation and formation of nuclei does not occur. For addition of pharmacological agents, trigger anaphase by adding 1/10 vol of a 10× calcium/agent stock and monitor as described previously. If anaphase is being monitored in real time, then add the agent from a 10× calcium/DAPI/agent stock. We have successfully used real-time analysis to monitor the effects of cyclin Δ90, AMPPNP, taxol, and vanadate on anaphase (Murray *et al.*, 1996; Desai *et al.*, 1998).

The more ambitious manipulation of depleting specific components and assaying the effect on chromosome segregation remains a technical challenge for the future. However, addition of reagents to anaphase reactions has been very useful, leading to the important discoveries that activation of the cyclin destruction machinery is sufficient to promote sister chromatid separation and that topoisomerase II activity is required for sister separation (Shamu and Murray, 1992; Holloway *et al.*, 1993).

VIII. Conclusions

In this chapter we have described detailed procedures for the preparation of spindle assembly extracts, for manipulation of extracts to define the function of specific proteins in spindle assembly, and for the analysis of anaphase chromosome movement *in vitro*. The importance of *Xenopus* extracts for analyzing spindle assembly and function is evident from their increasing use in recent years. However, many technical advances need to be made to allow a realization of the full potential of *Xenopus* extracts, particularly with respect to the manipulation and storage of extracts. We hope that the methods described here will serve as a starting point for future studies on spindle assembly and function and will stimulate technical innovations that will expand the types of analyses possible using *Xenopus* egg extracts.

Acknowledgments

We thank Manfred Lohka and Yoshio Masui, whose pioneering work led to the use of *Xenopus* egg extracts for studying spindle assembly and function; Manfred Lohka and James Maller, whose

early observations on *in vitro* spindles led to the development of the spindle assembly reactions described here; Ken Sawin, who characterized the pathways of spindle assembly and performed the first immunodepletions; and Caroline Shamu, who developed the *in vitro* anaphase reaction. We also thank Ted Salmon for his contributions to the analysis of anaphase *in vitro* and for many stimulating discussions on spindle assembly and chromosome movement. Finally, we thank our colleagues in the *Xenopus* extract field and members of our laboratories for many technical discussions on performing extract experiments. This work was supported by an HHMI predoctoral fellowship to A. D., grants from the NIH and Packard Foundation to A. M., grants from the NIH and HFSP to T. J. M. and postdoctoral fellowships from the NIH and the DOD Breast Cancer Research Program to C. E. W.

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